



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US95/09451 <b>(22) International Filing Date:</b> 18 July 1995 (18.07.95)  <b>(30) Priority Data:</b> 08/276,293      18 July 1994 (18.07.94)      US  <b>(71) Applicant:</b> DADE INTERNATIONAL INC. [US/US]; 1717 Deerfield Road, P.O. Box 778, Deerfield, IL 60015 (US).  <b>(72) Inventors:</b> HILYARD, Kathy, F.; 9840 Sheridan Street, Pembroke, FL 33024 (US). MONTICELLO, James; 2 Centenary Church Road, New City, NY 10956 (US). RUGG, James; 2015 N. Hibiscus Drive, North Miami, FL 33181 (US).  <b>(74) Agents:</b> PEARSON, Louise, S. et al.; 1717 Deerfield Road, P.O. Box 778, Deerfield, IL 60015 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** METHODS AND COMPOSITIONS FOR THE DETERMINATION OF RED BLOOD CELL FOLATE**(57) Abstract**

A composition is disclosed useful in the assay of folate from a hemolysate prepared from red blood cells using non-radiolabeled assay techniques such as fluorometric, colorimetric, enzymatic, or chemiluminescent assay methods. Methods to prepare hemolysates for the assay of folate are also disclosed.

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Methods and Compositions for the Determination of Red Blood  
Cell Folate

**Field of the Invention**

5       The present invention relates to methods and  
compositions useful in the determination of red cell folate.  
More particularly, the present invention relates to a method  
of sample preparation for the determination of the  
concentration of red cell folate using non-radiolabeled assay  
10 techniques and compositions useful for measuring the  
concentration of red cell folate using non-radiolabeled  
assays.

**Background of the Invention**

15       Measurement of folate in humans is important because  
folate deficiency is the predominant form of nutritional  
anemia. Other forms of nutritional anemia are vitamin B12  
deficiency and iron deficiency.

Folate levels may be measured in serum or red cells.  
20 Red cell folate concentrations are thought to show a better  
correlation with the presence of megaloblastic changes in  
erythrocytes than are folate serum levels. See, Measurement  
of Red cell Folate Levels by <sup>3</sup>H-Pteroylglutamic Acid (<sup>3</sup>H-  
PteGlu) Radioassay, Schreiber, Carol and Waxman, Samuel;  
25 British Journal of Haematology, (1974) 27, 551.

Currently, measurement of red cell folate levels is  
performed using radioassay techniques and in older  
microbiological techniques. In the radioassay techniques,  
whole blood is diluted with ascorbic acid in order to  
30 hemolyze the red blood cells. See, Measurement of low serum  
and red cell folate levels: a comparison of analytical

methods, Gilois, C.R. et al. Medical Laboratory Sciences (1987) 44, 33-40. Incubation is generally recommended and is usually about 90 minutes. Some procedures state that incubation is not required. In one procedure the hemolysate  
5 thus formed is further diluted with a protein diluent to mimic the concentration of protein found in standards. In other commercially available procedures, the hemolysate is used directly in the radioassay.

The hemolysates may then be run in radioassays for  
10 folate. Generally, the sample is combined with a known quantity of <sup>125</sup>I labeled folate with dithiothreitol and incubated. In some procedures which also assay for Vitamin B12 the solution is boiled. The mixture is then combined with folate binding protein. During an incubation step, the  
15 labeled folate competes with the folate in the sample for the folate binding protein. The solution is centrifuged and decanted. The red cell membrane is decanted off. The radioactivity of a pellet containing the folate is counted using standard techniques and the results compared to  
20 standards.

Radioassays suffer from the disadvantage that these assays use radioactive labels and the waste from these labels must be disposed of appropriately. These assays also have a number of long incubation steps.

25 Some commercially available radioassays include DPC DUALCOUNT SOLID PHASE NO BOIL ASSAY; Ciba Corning IMMOPHASE®; BECTON DICKINSON SimulTRAC-SNB Radioassay Kit; and BIO-RAD Quantaphase® Radioassay.

Development of non-radiolabeled assays for red cell  
30 folate, and particularly non-radiolabeled assays for red cell folate that utilize an absorbent solid phase, presents

a number of challenges. First, the red cells must be lysed so that the folate is released. Second, the folate must be stabilized. Third, the red cell membranes must be separated from the folate so that the membranes do not interfere with the measurement of folate. Separation by decanting, such as in the radioassay techniques would generally not be practical or sufficient for non-radiolabeled assay techniques.

The term non-radiolabeled assay as used herein means that the assay uses a label, but the label is not a radioactive label. Thus, the types of radioassays and microbiological assays are not included in this definition of non-radiolabeled assays. The types of assays that are non-radiolabeled include assays that use as a label, for example, a chromophore, a fluorophore, an enzyme, or a chemiluminescent molecule.

In particular, separation by decanting is impractical and insufficient for heterogeneous non-radiolabeled assays that utilize adsorbent solid supports such as paper, cellulose, polymers, or glass fibers. Generally in these methods, sample is added to a solid support which contains, or will contain, a binding protein (e.g. an antibody or folate binding protein) that is reactive to a molecule that selectively binds to the binding protein (e.g. an antigen or folate). There are many variations in these procedures that are known to those skilled in the art. Usually as a final step, the solid support is washed to remove unbound materials. However, red cell membranes are readily adsorbed by adsorbent solid supports and are difficult to remove. In addition, depending on the pore size of the solid support, the membranes may occlude the pores. This occlusion

prevents the flow of any wash solution and hence prevents the separation of unbound materials from bound materials. In addition, unwashed membrane may serve to bind enzyme conjugate leading to a higher background and depending on  
5 the signal molecule used in the assay, the membrane may contribute to the measured signal.

Currently there are commercially available non-radiolabeled based assays for the determination of serum folate. Examples of the available procedures include Baxter  
10 Diagnostics Inc. STRATUS® Folate Assay, a fluorometric enzyme assay and Ciba Corning ACS™ Folate Assay, a chemiluminescent assay. Currently, red cell folate can not be measured by these techniques. For example, the ACS™ Folate Assay procedure notes as a limitation that hemolysis  
15 significantly increases folate values due to the high folate concentration in red blood cells. The Stratus® Folate Assay procedures also states that hemolyzed samples must not be used. Thus, hemolyzed samples are to be avoided in non-radiolabeled assays.

20 Methods and compositions are needed to overcome the difficulties associated with radioassays for red cell folate and to make the determination of red cell folate practical and accurate using non-radiolabeled techniques. See, Red Cell Folate Assays: Some Answers to Current Problems with  
25 Radioassay Variability, Brown, Ross D. et al. Pathology 22 pp. 82-87 (1990).

#### Summary of the Invention

30 This invention provides a method of sample preparation for non-radiolabeled assay methods to measure red cell

folate. In these methods the red cell membrane is solubilized to provide for sufficient removal of the membrane from the solid phase. In addition, compositions are provided to solubilize red cell membranes.

5 In the method of the invention, an aliquot of whole blood is diluted with a hemolysing solutions such as ascorbic acid to form a hemolysate. The preparation of hemolysates is known in the art. Next, sufficient amounts of the hemolysate are combined with a diluent containing a  
10 detergent to solubilize the membranes. The hemolysate can now be analyzed by standard non-radiolabeled assay techniques to measure folate.

The composition of this invention is an aqueous solution comprising a buffer, a detergent that can  
15 solubilize red cell membranes contained in the hemolysate without the formation of a precipitate, and a salt. The composition may contain a folate-free inert protein and a preservative. Alternatively, the appropriate detergent can be added to buffered reagents that are already utilized in  
20 current serum folate assays.

#### Brief Description of the Drawings

Figure 1 shows patient sample results from an enzyme  
25 assay method for red cell folate of the present invention compared with a radioassay method.

Figure 2 shows patient sample results from an enzyme assay method for red cell folate of the present invention compared with a radioassay method.

Figure 3 shows patient sample results from an enzyme assay method for red cell folate of the present invention compared with a radioassay method.

Figure 4 shows patient sample results from an enzyme  
5 assay method for red cell folate of the present invention compared with a radioassay method.

#### Detailed Description of the Invention

The composition of the present invention comprises an  
10 aqueous solution of a detergent that can solubilize red cell membranes without the formation of a precipitate, a buffer, and a salt. The composition may contain a folate-free inert protein and a preservative.

The detergents of the present invention comprise those  
15 detergents that can solubilize red cell membranes and function in the final composition of the present invention to remove the membranes from the solid support. Preferably the solubilization occurs without the formation of a precipitate when the final composition containing the detergent is added  
20 to the hemolysate.

Detergents that are preferred include the zwitterionic detergents: (3-{3-cholamidopropyl} dimethyl-ammonio}-1-propanesulfonate) (hereinafter referred to as CHAPS), (3-[3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-propanesulfonate)  
25 (hereinafter referred to as CHAPS<sup>o</sup>), and N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate. N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate. Another detergent that was found to be acceptable was the non-ionic detergent n-Octyl- $\beta$ -D-glucopyranoside (OTG), however OTG was not  
30 preferred because slight prescription occurred when the final composition was added to the hemolysate.



The term acceptable, as used herein, means first that there is no more than a slight prescription precipitation when the composition of the present invention is added to a determined in non-radiolabeled assays of the present

5 invention are reasonably equivalent to those values that are obtained using commercially available radioassay methods is more than about 0.85, preferably more than about 0.90, and most preferably more than 0.95.

Detergents that were expected to work, but in fact did  
10 not work, were such non-ionic detergents as Tween-20, Brij-35, Nonidet P-40, Triton X-100, and POE coco amine. Although these detergents apparently solubilized the membranes (no precipitates were detected) these detergents were not acceptable. This was determined by substituting the above-  
15 mentioned detergents for detergents such as CHAPSO in the compositions of the present invention on thirteen patient blood samples. The results were compared to a radioassay method using the same thirteen patient blood samples. It was found that about 70% of the samples that were tested  
20 recovered red cell folate levels that were greater than two times the results recovered from the radioassay method.

Other non-ionic detergents that were tried included glucopon 425 CS which is (C<sub>6-12</sub> alkylmono and oligoglucopyranosides, and C<sub>10-16</sub> alkylmono and  
25 oligoglucopyranosides) and POE(5) soya amine. The glucopon 425 CS formed a heavy precipitate when added to the hemolysate and the POE(5) soya amine formed an oily mixture when combined with the other materials of the compositions

of the present invention. Thus, these detergents were unacceptable and were not evaluated in the enzyme assays for folate.

Other zwitterionic detergents that were tested but were  
5 found to be unacceptable were: N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate and N-octadecyl N,N-dimethyl-3-ammonio-1-propanesulfonate. The N-octyl-N,N-dimethyl-3-ammonio-1-propanesulfonate when used in the composition of the present invention formed a precipitate when combined  
10 with the hemolysate. Thus, the composition containing this detergent was not evaluated in the enzyme assays for folate. The N-octadecyl N,N-dimethyl-3-ammonio-1-propanesulfonate was insoluble in aqueous solution. Thus compositions of the present invention could not be prepared with this detergent.

15 It is important to note that all of the compositions containing zwitterionic detergents that did not form precipitates when combined with hemolysates were found to be acceptable and indeed are preferred detergents of the present invention.

20 Other detergents may be tested for acceptability by using the preferred embodiment of the compositions of the present invention (as described below), but substituting the preferred detergents for the detergent to be tested. First, the composition containing the detergent must not form more  
25 than a slight precipitate upon combining the composition with the hemolysate, preferably the final sample is clear to the eye. Second, results of patient samples as obtained in non-radiolabeled assays should be reasonable equivalent to those results from commercially available radioassay  
30 methods.

The concentration of the detergent should be at least about 0.5%. The preferred concentration range is between about 1 and 3%. The most preferred concentration is about 2%. Combinations of detergents are acceptable.

5       The buffers of the present invention include, but are not limited to, TRIS, borate buffers, sodium or potassium phosphate buffers, 3-[N,N-bis(2-Hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid (DIPSO), 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxy-propanesulfonic  
10   acid] (TAPSO), N-[2-hydroxyethyl]piperazine-N'-[2-hydroxy-propanesulfonic acid] (HEPPSO), Piperazine-N,N'-bis[2-hydroxypropanesulfonic acid] (POPSO), N-[2-hydroxyethyl][piperazine-N'-[3-propanesulfonic acid] (EPPS),  
15   Triethanolamine (TEA), Tricine, Bicine, and N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; ([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)-1-propanesulfonic acid) (TAPS).

The choice of buffer is not critical, however the preferred buffers are those buffers that have a pH buffering  
20   capacity at between about pH 8 to 10. The buffering capacity at this pH range is important because the assays for serum folate are conducted at about pH 9 to 9.5. The most preferred buffer is TRIS because of its availability. The preferred pH range is about 8 to 10 and most preferred  
25   is 8.5 to 9.5.

Buffers were tested at a variety of concentrations. The concentration of buffer was found not to be critical although it was found that some level of buffer was  
30   required. A composition which did not include a buffer gave folate results that were highly variable when one sample was repeatedly tested in an enzyme assay. The concentration of

the buffer may be from about 0.01 M to 1M. The preferred concentration of the buffer is about 50 mM to 200 mM.

A salt may also be added to the compositions. The salts that may be added to the compositions of the present invention include, but are not limited to, sodium chloride, potassium chloride, sodium sulfate, sodium bromide, potassium bromide, calcium chloride, barium sulfate, barium chloride, and lithium chloride. The most preferred salt is sodium chloride because of its availability and because of its compatibility with blood.

The concentration of the salt may be from about 0 to over 0.5 M. The preferred concentration is physiological concentrations of about 0.85%.

An inert protein may also be added to the composition. If protein is added to the composition, it is important that it is folate-free. The protein can be recombinant or native. The proteins that may be added include, but are not limited to, albumin, gelatin, ovalbumin. Bovine serum albumin is preferred because of its availability.

The protein may be added in concentrations from about 0 to over 10%. The preferred amount of protein may be required in order to obtain correct results for samples that have a low hematocrit, however a study of a limited number of samples that have a low hematocrit demonstrated that the protein did not affect the folate values.

For commercial embodiments, a preservative should be added to prevent the growth of bacteria and/or fungi. Common preservatives include sodium azide, gentamicin, nystatin, thimerosal, and kathon.

The compositions of the present invention may be prepared by combining effective amounts of detergent in buffer. The composition may include effective amounts of salt, protein and preservative. For example, a preferred composition was prepared as follows: To prepare one liter combine about 50 grams of folate-free bovine serum albumin to about 800 milliliters of a 50mM solution of TRIS and 0.85% sodium chloride (TRIS Buffered Saline). The albumin was allowed to dissolve using gentle mixing while avoiding foaming. About 4 milliliters of a 25% solution of sodium azide was added while the solution was mixing. About 20 grams of CHAPS was added while the solution was mixing. Mixing was continued until the CHAPS was completely dissolved. The pH of the solution was adjusted to about 9.3 and the volume was adjusted to 1 liter with TRIS Buffered Saline. The solution was sterile filtered. It is important to note that the order of addition of the reagents is not important. Generally, the protein is added first because it takes the longest to dissolve.

In the method of the present invention, a composition is prepared as described above. Hemolysate samples are combined with the composition. The diluted hemolysate is assayed using commercially available non-radiolabeled assays kits for serum folate.

Alternatively, currently available assay kits contain reagents such as neutralizing or conditioning reagents. These neutralizing or conditioning reagents are appropriately buffered. For example, the ACS™ Folate Assay contains conditioning reagents such as the Folate Conditioning Reagent B which contains a BSA buffered solution and the Stratus® Folate Assay contains a Folate

Neutralizer Reagent which is a tetraborate buffered solution. Thus, the detergents of the present invention can be added to these reagents and the sample can be assayed using the kit.

5 Hemolysate samples are prepared by making a 1:10 dilution to 1:50 dilution of whole blood with a solution of about 0.125% to 2% ascorbic acid solution. The preparation of hemolysate samples is known in the art. See, Measurement of low serum and red cell folate levels: a comparison of  
10 analytical methods, Gilois, C.R. et al. Medical Laboratory Sciences (1987) 44, 33-40. For instance, in the Bio-Rad Red Cell Folate Reagent Pack in combination with the Bio-Rad Quantaphase® B12/Folate Radioassay use a 1:11 dilution of blood to a 0.4% ascorbic acid solution and the DPC DUALCOUNT  
15 B12/Folate No Boil Assay requires that whole blood be diluted 1:21 with a 1% solution of ascorbic acid.

The hemolysate may be used fresh or stored frozen. If the hemolysates are to be used fresh an incubation period from about 0 to over 90 minutes at room temperature may be  
20 performed to ensure that the whole blood is completely hemolysed. The preferred incubation time is 30-90 minutes, and the most preferred incubation time is about 90 minutes.

In the method of the present invention, approximately equal amounts of the hemolysate are combined with the  
25 compositions of the present invention. Then the mixture is assayed in available non-radiolabeled assay techniques such as assay methods that utilize enzymes, colorimetric molecules, chemiluminescent molecules and fluorometric molecules as labels.

30 It is believed that the method to prepare samples for non-radiolabeled assays is most beneficial, but is not

limited to, those assays that utilize absorbent solid phases listed above. Examples of the types of assays that use such solid phases include Baxter Diagnostics Inc. STRATUS® FLUOROMETRIC ASSAY SYSTEM; PB Diagnostic Inc. OPUS® Assay System and Abbott's ImX® Assay System.

The invention can be further understood by reference to foregoing examples which are for illustration only and are not to be construed as a limitation on the invention.

#### 10 Example 1

##### Preparation of Hemolysate Diluent

I. Materials: TRIS: molecular weight 121.1

NaCl: molecular weight 58.44

CHAPS: Sigma Chemical Co.

15 Folate-free BSA: Interger Inc.

##### II. Preparation of Stock Solutions

A. A stock solution of 20% folate-free BSA (FFBSA) and 8% CHAPS was prepared as follows: Twenty grams of FFBSA were added to 90 milliliters of distilled water and stirred gently until the FFBSA was completely dissolved. Eight grams of CHAPS were added to the FFBSA solution and stirred gently until the CHAPS was dissolved. The total volume was brought up to 100 milliliters with distilled water.

(Stock A)

B. A stock solution of 2 M TRIS was prepared by adding 48.44 grams of TRIS to about 90 milliliters of distilled water and was stirred until the TRIS was dissolved. The

total volume was brought up to 200 milliliters with distilled water. (Stock B)

C. A stock solution of 4 M NaCl was prepared by adding 23.38 grams of NaCl to about 90 milliliters of distilled water and stirred until the salt was dissolved. The total volume was brought up to 200 milliliters with distilled water. (Stock C)

### III. Preparation of the Hemolysate Diluent

One milliliter of Stock B and 1.5 milliliters of Stock C were added to 10 milliliters of Stock A. The pH of the solution was adjusted to 9.30 +/- .05. The volume of the solution was brought up to about 40 milliliters and the pH was rechecked. Final concentration of the diluent is about 50 mM TRIS, 150 mM NaCl, 2% CHAPS, and 5% FFBSA.

### Example 2

#### Assay of Folate in Red Blood Cells and Comparison with Radioassay Methods

Fifty patient samples were prepared for assay of folate using a non-radiolabeled assay, the Stratus® Folate assay and compared with two different radio-labeled assays, the DPC DUALCOUNT B12/Folate No Boil Assay and the BIO-RAD Quantaphase® B12/Folate Radioassay.

25

#### 1. Preparation of Hemolysate Diluent

A. Materials: TRIS: molecular weight 121.1  
NaCl: molecular weight 58.44  
CHAPS: Sigma Chemical Co.  
Folate-free BSA (FFBSA): Intergen Inc.

30



B. A solution of 100 mM TRIS was prepared by dissolving 12.11 grams of TRIS in 1 liter of distilled water. About 8.76 grams of NaCl was added to an aliquot of 500 mls of the TRIS buffer to form 100 mM TRIS with 300 mM NaCl (TRIS  
5 Buffered Saline). Sodium azide was added to a final concentration of 0.2%.

About 0.25 grams of FFBSA were added to 50 milliliters of the TRIS Buffered Saline. The solution was stirred gently until dissolved to form TRIS Buffered Saline-BSA. Next about  
10 0.5 grams of CHAPS were added to 25 milliliters of the TRIS Buffered Saline-BSA. The pH was adjusted to about 9.3. The final concentration is 100 mM TRIS, 300 mM NaCl, 0.5% BSA, 2% CHAPS and 0.2% Sodium Azide.

15 II. Preparation of Hemolysate from Blood

A. For the DPC folate assay an aqueous solution of 1.0% Ascorbic Acid (Aldrich) was prepared. One hundred microliters of blood was added to 2 milliliters of the  
20 ascorbic acid solution. The samples were stored frozen at -20C until used in the assay in performed.

B. For the BIO-RAD folate assay an aqueous solution of 0.4% Ascorbic Acid (BIO-RAD) was prepared. One hundred microliters of blood from each sample was combined with 1  
25 milliliter of the 0.4% ascorbic acid solution. The samples are stored frozen at -20 C until the assay was performed.

C. For the Stratus folate assay an aqueous solution of 0.4% Ascorbic Acid (Aldrich) was prepared. One hundred microliters of blood from each sample was combined with 1  
30 milliliter of the 0.4% ascorbic acid solution. The samples are stored frozen at -20 C until the assay was performed.

### III. Preparation of Samples for Assay from Hemolysates

- 5 A. For the DPC assay, the hemolysates were thawed and no further processing was required.
- B. For the Bio-Rad assay, the hemolysates were thawed and 100 microliters of hemolysate was added to 100 microliters of Bio-Rad protein diluent.
- 10 C. For the Stratus assay, the samples were thawed and 100 microliters of the hemolysates were diluted with one hundred microliters of the Hemolysate Diluent, prepared as described in Example 2 Section I.

### IV. Assay of the Samples

- 15 A. The samples as prepared in Example 2 Section IIIA, were assayed according to the package insert for the DPC assay. Appropriate controls were analyzed for the assay.
- B. The samples as prepared in Example 2 Section IIIB, were assayed according to the package insert for the Bio-rad
- 20 assay. Appropriate controls were analyzed for the assay.
- C. The samples as prepared in Example 2 Section IIIC, were assayed according to the package insert for the Stratus assay. Each sample was assayed using two different Stratus kit lots. Appropriate controls were analyzed for the assay.

25

### V. Results

Sample #	HCT	Bio-Rad	DPC	Stratus	Stratus
1	23	478	584	723	669
2	31	390	427	490	454

3	39	350	538	355	327
4	23	373	347	507	459
5	28	196	195	346	314
6	44	260	535	395	314
7	35	352	444	515	484
8	43	261	352	353	327
9	22	240	277	460	410
10	34	1191	1365		
11	26	330	355	372	338
12	27	652	747	904	847
13	51	315	325	418	393
14	25	475	470	554	510
15	47	407	456	505	477
16	35	817	762	867	817
17	21	262	290	566	513
18	33	507	490	633	593
19	48	426	429	522	495
20	32	481	1628	591	550
21	30	924	910	917	865
22	45	220	229	283	259
23	37	244	278	345	309
24	46	512	575	574	540
25	38	399	608	481	446
26	42	262	340	325	298
27	49	224	244	256	233
28	18	831	805	941	868
29	39	214	226	293	265
30	22	260	248	440	390
31	30	301	315	403	367
32	24	431	534	541	495
33	32	234	236	323	289

18

34	19	313	309	509	451
35	38	515	492	660	625
36	29	303	442	387	349
37	31	362	426	475	433
38	50	453	458	497	466
39	52	144	161	199	182
40	51	397	370	449	418
41	50	290	302	326	304
42	33	667	706	587	547
43	50	576	596	647	612
44	34	298	371	362	330
45	36	495	630	550	513
46	41	762	953	901	858
47	21	765	950	932	869
48	36	79	93	208	177
49	20	1430	1187	1595	1518
50	37	529	868	589	553

Table 1

Results are presented graphically in Figures 1-4.

5

### Example 3

#### I. Preparation of Hemolysate Diluent

A. Materials: TRIS: molecular weight 121.1

NaCl: molecular weight 58.44

CHAPS: Sigma Chemical Co.

10

Folate-free BSA: Intergen Inc.

B. A solution of 100 mM TRIS is prepared by dissolving 12.11 grams of TRIS in 1 liter of distilled water. About 8.76 grams of NaCl is added to an aliquot of 500 mls of the

TRIS buffer to form 100 mM TRIS with 300 mM NaCl (TRIS Buffered Saline). Sodium azide is added to a final concentration of 0.2%.

About 0.29 grams of FFBSA are added to 50 milliliters of the  
5 TRIS Buffered Saline. The solution is stirred gently until dissolved to form TRIS Buffered Saline-BSA. Next about 0.5 grams of CHAPS are added to 25 milliliters of the TRIS Buffered Saline-BSA. The pH is adjusted to about 9.3. The final concentration is 100 mM TRIS, 300 mM NaCl, 0.5% BSA, 2%  
10 CHAPS and 0.2% Sodium Azide.

## II. Preparation of Hemolysates from Blood

A. For the Ciba-Corning ACS™ folate assay an aqueous solution of 0.4% Ascorbic Acid (Aldrich) is prepared. One  
15 hundred microliters of blood from each sample is combined with 1 milliliter of the 0.4 ascorbic acid solution. The samples are stored frozen at -20 C until the assay is performed.

## 20 III. Preparation of Samples for Assay from Hemolysates

A. For the ACS assay, the samples are thawed and 100 microliters of the hemolysates are diluted with one hundred microliters of the Hemolysate Diluent, prepared as described in Example 3 Section I.

25

## IV. Assay of the Samples

A. The samples as prepared in Example 3 Section IIIA, are assayed according to the package insert for the ACS assay. Appropriate controls are analyzed for the assay.

30

Example 4

The effects of adding protein to the diluent were investigated.

I. Preparation of Hemolysate Diluent

- 5 A. Materials: TRIS: molecular weight 121.1  
NaCl: molecular weight 58.44  
CHAPS: Sigma Chemical Co.  
Folate-free BSA: Intergen Inc.

10 B. Preparation of Diluent.

Control diluent was prepared as described in Example 2 I B.

- Protein free diluent was prepared as follows: About 0.30275 grams of TRIS and 0.4383 grams of NaCl was added to about 20 milliliters of distilled water. The pH was adjusted to 9.3 About 1 gram of CHAPS was added and the total volume was adjusted to about 40 milliliters. The pH was rechecked. No adjustment was needed. The total volume was adjusted to 50 mls.

20

II. Preparation of Hemolysates from Blood

- A. An aqueous solution of 0.4% Ascorbic Acid (Aldrich) was prepared. One hundred microliters of blood from each sample was combined with 1 milliliter of the 0.4% ascorbic acid solution. The samples were stored frozen at -20 C until the assay was performed.

III. Preparation of Samples for Assay from Hemolysates

- A. The samples were thawed and 100 microliters of the hemolysates were diluted with one hundred microliters of the

30

Hemolysate Diluents - one with protein and one without protein, prepared as described in Example 4 Section I.

#### IV. Assay of the Samples

- 5 A. The samples as prepared in Example 4 Section IIIA, were assayed according to the package insert for the Stratus assay. Appropriate controls were analyzed for the assay.

#### V. Results

10

Sample	Control Diluent	Protein-Free Diluent	Observed/ Expected
1	200	220	110%
2	220	250	114%
3	187	201	107%
4	429	419	96%

Table 2

Protein is not critical for the diluent.

15

#### Example 5

Effect of Different Detergents on the Diluent

##### I. Preparation of Hemolysates

- 20 Hemolysates were prepared on two different samples as described in Example 4, II A and were incubated for 90 minutes in the dark prior to use.

##### II. Preparation of Diluent

Different detergents were added at a final concentration of 1% to a Stratus Neutralizing Reagent- a solution of sodium tetraborate buffer at pH 8.0 with 0.1% sodium azide.

5     III. Preparation of Samples from Hemolysates

Two different samples were diluted 1:1 in a TRIS buffered saline with 5% protein based diluent containing 1% NP-40.

IV. Assay of Samples

10    A Stratus Folate Assay kit was used to evaluate the samples. The neutralizing reagents prepared as described in Example 5, II were substituted for the commercial Neutralizing Reagent. The commercial Neutralizing Reagent was used as a control. In every other respect, the assay was conducted as per the  
15    manufacturer's instructions.

V. Results

Neutralizing Agent with detergent	Sample 1	Sample 2
Control (no detergent)	7.2	13.0
1% Triton	6.6	15.8
1% NP-40	8.9	15.3
1% Tween-20	6.9	14.7
1% CHAPSO	5.0	6.7
1% BRIJ-35	7.0	14.4



Only CHAPSO was able to reduce the folate levels to expected results.

#### Example 6

#### 5 Comparison of CHAPS and CHAPSO Detergents

##### 1. Preparation of Hemolysate Diluents

A. Materials: TRIS: molecular weight 121.1  
NaCl: molecular weight 58.44  
10 CHAPS: Sigma Chemical Co.  
Folate-free BSA: Intergen Inc.  
CHAPSO

##### B. Preparation of Diluent.

15 Ten diluents were prepared as described in Example 2 I  
B. The difference between the diluents were as follows: One  
diluent contained 1% CHAPSO and 0.5% BSA, the second diluent  
contained 1% CHAPS AND 0.5% BSA, THE THIRD DILUENT CONTAINED  
2% CHAPSO AND 0,5% BSA, THE FOURTH DILUENT CONTAINED 2% chaps  
20 and 0.5% BSA, the fifth diluent contained 1% CHAPSO and 5%  
BSA, the sixth diluent contained 1% CHAPS and 5% BSA, the  
seventh diluent contained 2% CHAPSO and 5% BSA, the eighth  
diluent contained 2% CHAPS and 5% BSA, the ninth diluent  
contained 1% CHAPSO and 1% CHAPS and 0.5% BSA and the tenth  
25 contained 1% CHAPSO and 1% CHAPS and 5% BSA. The control  
contained 1% NP-40 and 5% BSA.

##### II. Preparation of Hemolysates

Hemolysates were prepared on two different samples, which  
30 were particularly problematic, as described in Example 4, II

A and were incubated for 90 minutes in the dark prior to use.

### III. Preparation of Samples

Two samples were combined 1:1 with each of the different  
5 diluents.

IV. The samples were assayed in a Stratus Assay for Folate following the manufacturer's directions.

### 10 V. Results

Diluent	Sample 1	Sample 1/ Bio-rad	Sample 2	Sample 2/ Bio-rad
Control	578	1.49	810	2,31
Control	542	1.40	835	2.39
1% CHAPSO	422	1.09	575	1.64
0.5% BSA				
2% CHAPSO	335	0.87	406	1.16
0.5% BSA				
1% CHAPSO	388	1.00	523	1.49
5% BSA				
2% CHAPSO	338	0.87	440	1.26
0.5% BSA				
1% CHAPSO	330	0.85	415	1.19
1% CHAPS				
1% CHAPS	423	1.09	777	2.22
0.5% BSA				
CONTROL				
CONTROL	537	1.39	801	2.29
1% CHAPS	388	1.00	608	1.74
0.5% BSA				

2% CHAPS	314	0.81	413	1.18
0.5% BSA				
1% CHAPS	301	0.78	590	1.69
5% BSA				
2% CHAPS	296	0.76	462	1.32
5% BSA				
1% CHAPS	272	0.70	414	1.18
1% CHAPSO				
5% BSA				

CHAPS and/or CHAPSO at 2% is best for the samples.

#### 5 Example 7

The effects of buffer concentration and salt concentrations were determined.

#### 1. Preparation of Hemolysate Diluents

- 10 The stock solutions described in Example 1 were used to prepare a variety of diluents. The concentrations of each reagent are as follows:

Diluent #	[TRIS] mM	[NaCl] mM	BSA %	CHAPS %
1	1000	500	5	2
2	505	500	5	2
3	10	500	5	2
4	1000	0	5	2
5	505	0	5	2
6	505	1000	5	2
7	1000	1000	5	2
8	10	1000	5	2

26

9	10	0	5	2
10 (CNTRL)	50	150	5	2

## II. Preparation of Hemolysates

Hemolysates were prepared on a single sample, as described  
5 in Example 4, II A and were incubated for 90 minutes in the  
dark prior to use.

## III. Preparation of Samples

Two samples were combined 1:1 with each of the ten different  
10 diluents.

IV. The samples were assayed in a Stratus Assay for Folate  
following the manufacturer's directions.

## 15 V. Results

Diluent #	Result	ratio of experimental/ control
1	220	0.92
2	199	1.02
3	195	1.04
4	239	0.85
5	228	0.89
6	152	1.34
7	170	1.19
8	175	1.16
9	212	1.04
10	203	1

The amount of salt and TRIS does not have an effect on the diluent. TRIS may be used at a range from 0.01 M to 1 M and NaCl may be used at a range from 0 to at least about 0.5

5 M.

We claim:

1. A composition useful for the determination of folate  
from a hemolysate of red blood cells in a non-radiolabeled  
5 assay comprising:
  - a) a detergent; and
  - b) a buffer;wherein the composition solubilizes membranes of red blood  
cells in the hemolysate and allows for the determination of  
10 folate in said non-radiolabeled assay.
2. The composition of claim 1 further comprising a salt.
3. The composition of claim 1 further comprising an inert  
15 protein.
4. The composition of claim 1 wherein the label of the  
non-radiolabeled assay is selected from the group consisting  
of enzymes, colorimetric molecules, fluorometric molecules  
20 and chemiluminescent molecules.
5. The composition of claim 1 wherein the detergent is  
selected from the group consisting of (3-{3-  
cholamidopropyl}dimethyl-ammonio)-1-propanesulfonate), (3-  
25 [3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-  
propanesulfonate), and N-dodecyl-N,N-dimethyl-3-ammonio-1-  
propanesulfonate.
6. The composition of claim 1 wherein the buffer has a  
30 buffering capacity from pH 8 to pH 10.

7. The composition of claim 1 wherein the buffer is selected from the group consisting of TRIS, borate buffers, sodium or potassium phosphate buffers, 3-[N,N-bis(2-Hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid (DIPSO),  
5 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxy-propanesulfonic acid] (TAPSO), N-[2-hydroxyethyl]piperazine-N'-[2-hydroxy-propanesulfonic acid] (HEPPSO), Piperazine-N,N'-bis[2-hydroxypropanesulfonic acid] (POPSO), N-[2-hydroxyethyl][piperazine-N'-[3-propanesulfonic acid] (EPPS),  
10 Triethanolamine (TEA), Tricine, Bicine, and N-tris[hydroxymethyl)methyl-3-aminopropanesulfonic acid; ([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)amino]-1-propanesulfonic acid) (TAPS).
- 15 8. The composition of claim 2 wherein the salt is selected from the group consisting of sodium chloride, potassium chloride, sodium sulfate, sodium bromide, potassium bromide, calcium chloride, barium sulfate, barium chloride, and lithium chloride.
- 20 9. The composition of claim 3 wherein the inert protein is selected from the group consisting of albumin, ovalbumin and gelatin.
- 25 10. A composition useful for the determination of folate from a hemolysate of red blood cells in a non-radiolabeled assay comprising an aqueous solution of:
- 30 a) a zwitterionic detergent that solubilizes red cell membranes;
- b) a buffer wherein the buffer has a buffering capacity from pH 8 to 10;

- c) a salt; and
  - d) an inert protein selected from the group consisting of albumin, ovalbumin, and gelatin.
- 5 11. The composition of claim 10 wherein the buffer is TRIS, the salt is sodium chloride, and the protein is bovine serum albumin.
12. A composition useful for the determination of folate
- 10 from a hemolysate of red blood cells in a non-radiolabeled assay comprising:
- a) a detergent selected from the group consisting of (3-{3-cholamidopropyl}dimethyl-ammonio)-1-propanesulfonate), (3-{3-cholamidopropyl}-dimethylammonio-2-hydroxy-1-
  - 15 propanesulfonate), and N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate;
  - b) a buffer wherein the buffer has a buffering capacity from pH 8 to 10;
  - c) a salt; and
  - 20 d) an inert protein selected from the group consisting of albumin, ovalbumin, and gelatin.
13. The composition of claim 12 wherein the buffer is TRIS, the salt is sodium chloride, and the protein is bovine serum
- 25 albumin.
14. A method to prepare a hemolysate for the determination of folate in red cells comprising:
- a. combining an effective amount of the composition of
  - 30 claim 1 and the hemolysate to form a mixture; and



b. assaying an aliquot of the mixture in a non-radiolabeled assay.

15. The method of claim 14 wherein the label of the non-radiolabeled assay is selected from the group consisting of colorimetric molecules, fluorometric molecules, enzymes and chemiluminescent molecules.

16. The method of claim 15 wherein the non-radiolabeled assay utilizes a solid phase and said solid phase is a porous matrix wherein the material of the porous matrix is selected from the group consisting of cellulose, paper, and glass fiber.

17. A method to prepare a hemolysate for the determination of folate in red cells comprising:

- a. combining an effective amount of the composition of claim 10 and the hemolysate to form a mixture; and
- b. assaying an aliquot of the mixture in a non-radiolabeled assay.

18. The method of claim 17 wherein the label of the non-radiolabeled assay is selected from the group consisting of colorimetric molecules, fluorometric molecules, enzymes and chemiluminescent molecules.

19. The method of claim 18 wherein the non-radiolabeled assay utilizes a solid phase and the solid phase is a porous matrix wherein the material of the porous matrix is selected from the group consisting of cellulose, paper, and glass fiber.

20. A method to prepare a hemolysate for the determination of folate in red cells comprising:
- a. combining an effective amount of the composition of claim 12 and the hemolysate to form a mixture; and
  - b. assaying an aliquot of the mixture in a non-radiolabeled assay.
21. The method of claim 20 wherein the label of the non-radiolabeled assay is selected from the group consisting of colorimetric molecules, fluorometric molecules, enzymes and chemiluminescent molecules.
22. The method of claim 21 wherein the non-radiolabeled assay utilizes a solid phase and the solid phase is a porous matrix wherein the material of the porous matrix is selected from the group consisting of cellulose, paper, and glass fiber.

1/4

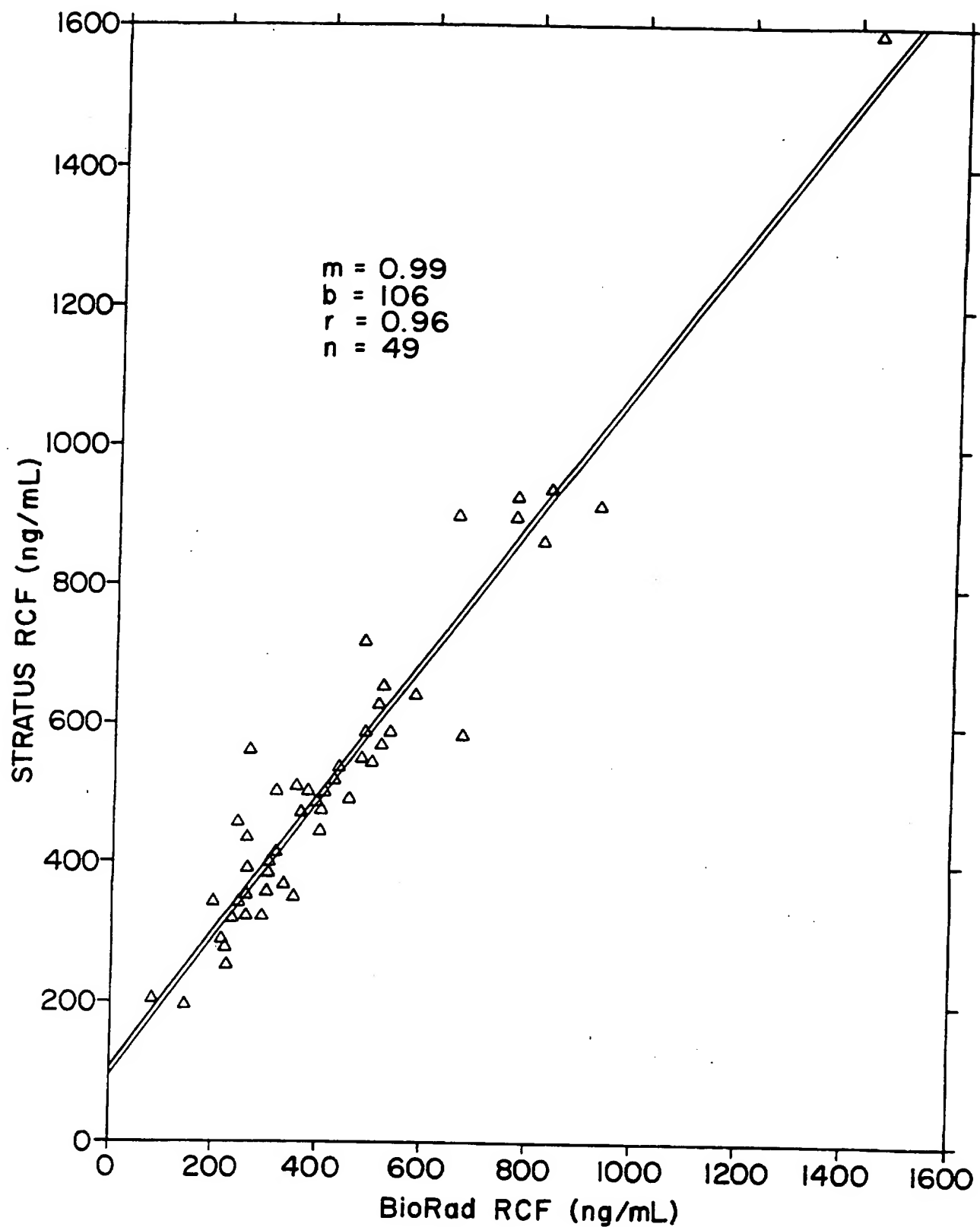
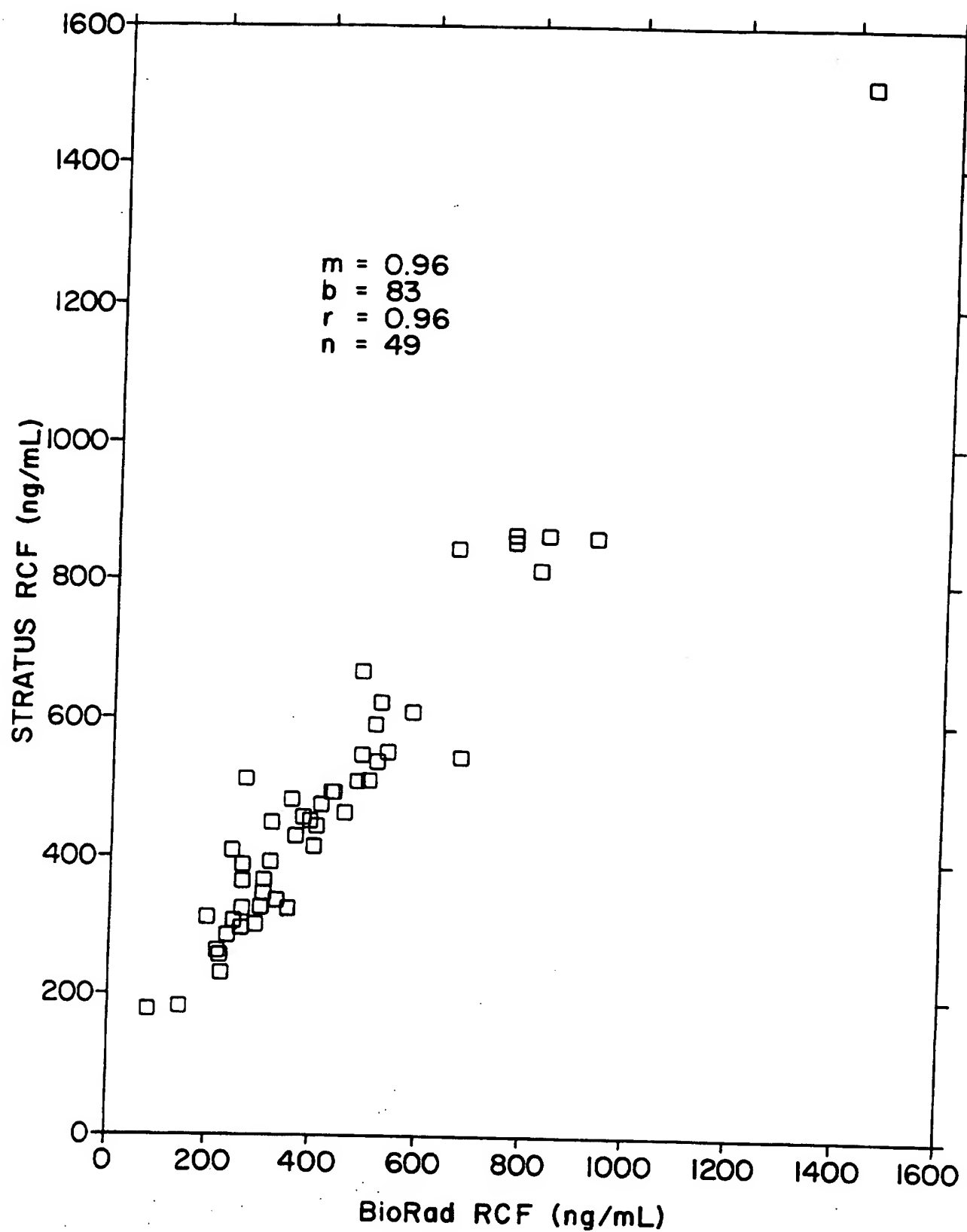
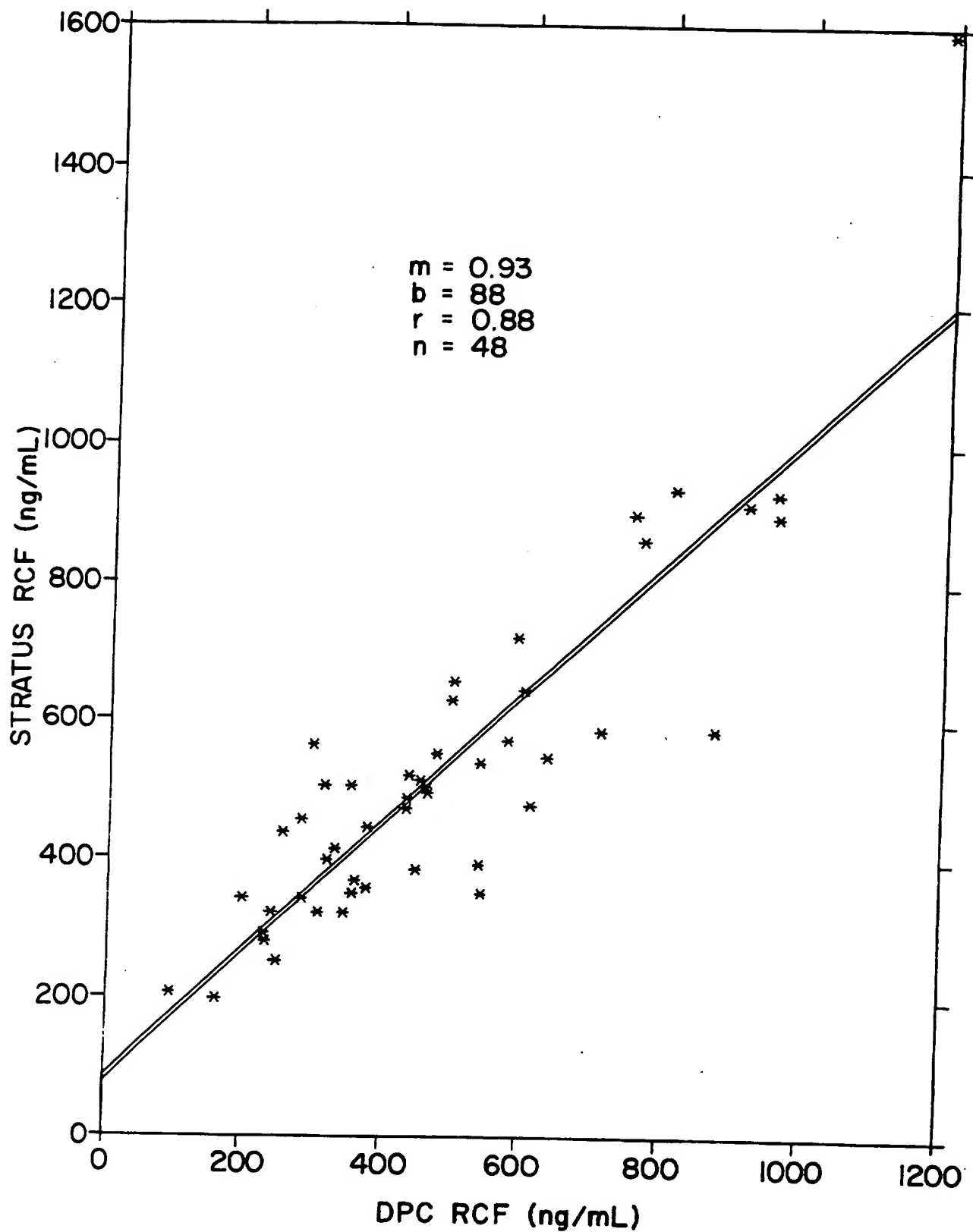


FIG. 1

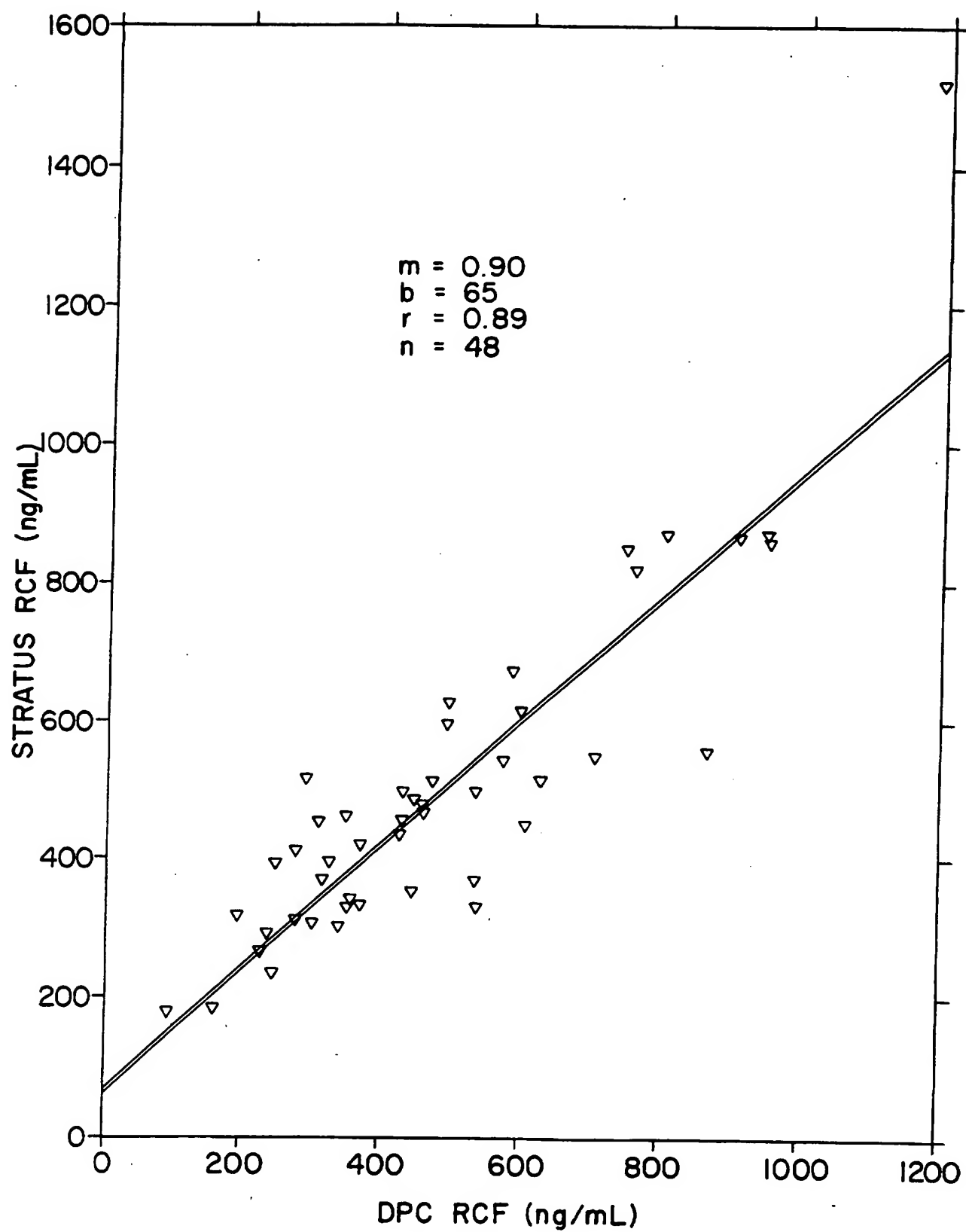
2/4

**FIG. 2**

3/4

**FIG. 3**

4 / 4

**FIG. 4**

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/09451

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/82 G01N33/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLINICAL CHEMISTRY, vol. 28, no. 1, January 1982 WINSTON US, pages 117-118, S. I. HANSEN ET AL. 'Detergent activation of the binding protein in the folate radioassay.'  --- -/--	

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 107, no. 19, 9 November 1987 Columbus, Ohio, US; abstract no. 173272, A. C. ANTONY ET AL. 'Identification of high affinity folate binding proteins in human erythrocyte membranes' page 479; column 2; see abstract & J. CLIN. INVEST., vol. 80, no. 3, 1987 pages 711-723, -----	
A	US,A,4 806 493 (A. YUAN.) 21 February 1989 -----	
A	ANALYTICAL CHEMISTRY, vol. 56, no. 9, August 1984 COLUMBUS US, pages 1723-1726, L. G. BACHAS ET AL. 'Cooperative interaction of immobilised folate binding protein with enzyme-folate conjugates: An enzyme-linked assay for folate.' -----	
A	EP,A,0 382 334 (BIO-RAD LABORATORIES, INC.) 16 August 1990 -----	



Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4806493	21-02-89	NONE	
EP-A-382334	16-08-90	CA-A- 2008473 JP-A- 2247566	09-08-90 03-10-90

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